Biochimica et Biophysica Acta 1840 (2014) 1651–1656

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Comparison of quantum dot-binding protein tags: Affinity determination by ultracentrifugation and FRET

Mara Werwie [a], Niklas Fehr [a], Xiangxing Xu [b,1], Thomas Basché [b], Harald Paulsen [a,*]

[a] Institut für Allgemeine Botanik, Johannes-Gutenberg-Universität Mainz, Johannes-von-Müllerweg 6, 55099 Mainz, Germany
[b] Institut für Physikalische Chemie, Johannes-Gutenberg-Universität Mainz, Duesbergweg 10-14, 55128 Mainz, Germany

ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form 7 November 2013
Accepted 25 November 2013
Available online 19 December 2013

Keywords:
Nanoparticle
Protein
Stoichiometry
Ultracentrifugation
Fluorescence resonance energy transfer (FRET)
Light-harvesting complex II (LHCII)

ABSTRACT

Background: Hybrid complexes of proteins and colloidal semiconductor nanocrystals (quantum dots, QDs) are of increasing interest in various fields of biochemistry and biomedicine, for instance for biolabeling or drug transport. The usefulness of protein–QD complexes for such applications is dependent on the binding specificity and strength of the components. Often the binding properties of these components are difficult and time consuming to assess.
Methods: In this work we characterized the interaction between recombinant light harvesting chlorophyll *a*/*b* complex (LHCII) and CdTe/CdSe/ZnS QDs by using ultracentrifugation and fluorescence resonance energy transfer (FRET) assay experiments. Ultracentrifugation was employed as a fast method to compare the binding strength between different protein tags and the QDs. Furthermore the LHCII:QD stoichiometry was determined by separating the protein–QD hybrid complexes from unbound LHCII via ultracentrifugation through a sucrose cushion.
Results: One trimeric LHCII was found to be bound per QD. Binding constants were evaluated by FRET assays of protein derivatives carrying different affinity tags. A new tetra-cysteine motif interacted more strongly ($K_a = 4.9 \pm 1.9\ nM^{-1}$) with the nanoparticles as compared to a hexahistidine tag (His_6 tag) ($K_a \sim 1\ nM^{-1}$).
Conclusion: Relative binding affinities and binding stoichiometries of hybrid complexes from LHCII and quantum dots were identified via fast ultracentrifugation, and binding constants were determined via FRET assays.
General significance: The combination of rapid centrifugation and fluorescence-based titration will be useful to assess the binding strength between different types of nanoparticles and a broad range of proteins.

1. Introduction

Colloidal semiconductor nanocrystals or quantum dots (QDs) are widely used for labeling biological components [1–3]. The advantages of QDs compared to fluorescent dyes include their high photostability and their tunable, size dependent absorption and fluorescence emission bands. Their strong fluorescence makes them excellent candidates for fluorescence resonance energy transfer (FRET) studies for example with fluorescent-dye labeled biomaterials [4–6]. As a further advantage, QD–protein (or peptide) conjugates often form spontaneously and several strategies have been described for this self-assembly. Poly histidine tags chelate metal ions on the surface of ZnS-coated core/shell QDs [7–11]. A strong affinity to ZnS surfaces was also observed with peptides containing multiple repeats of cysteine pairs [12]. Furthermore, electrostatic interactions between charged ligands on QD surfaces and protein domains [13,14] or membrane surfaces [15] carrying the opposite charges have been employed for complex formation.

If QDs are to be applied as protein labels, it is helpful to know the QD–protein stoichiometry or to establish strategies to make hybrid complexes at defined ratios. Hybrid complexes of QDs and polyhistidine-tagged proteins at different stoichiometries were separated by gel electrophoresis [10]. This method has also been used in combination with Western blotting, to approximate the average stoichiometries of antibody conjugated QDs [16]. The protein–QD stoichiometry in hybrid complexes has been (roughly) estimated by analytical ultracentrifugation [17]. In another approach the formation of protein–QD assemblies at a 1:1 ratio was revealed by atomic force microscopy [18]. All methods have in common that they are rather complex and/or can give only a rough estimate of the QD–protein stoichiometry.

Here we present a very simple and fast method to assess different protein affinity tags with regard to their efficiency in promoting QD–protein binding and to average QD–protein stoichiometries. By using rapid ultracentrifugation, QD–protein complexes were separated from unbound protein. FRET assays were used to estimate the binding constants of different binding tags.

In the experiments described here, type-II CdTe/CdSe/ZnS QDs were used that were functionalized with dihydrolipoic acid (DHLA) for water-solubility. The protein component to be bound to QDs was recombinant light harvesting complex II (LHCII), reconstituted in vitro

* Corresponding author.
E-mail address: paulsen@uni-mainz.de (H. Paulsen).
[1] Present address: State Key Laboratory of Coordination Chemistry, School of Chemistry and Chemical Engineering, Nanjing University, Hankou Road 22, Nanjing, CN 210093, People's Republic of China.

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.11.025

from its bacterially expressed apoprotein and plant pigments (14 chlorophyll and 4 carotenoid molecules per protein) [19]. The recombinant nature of the protein opens up the possibility of introducing different affinity tags for comparison, without altering the protein structure otherwise. The chlorophylls bound to LHCII are endogenous fluorophores that can be used to monitor protein binding to QDs by serving as acceptors of the QD excitation energy [14].

2. Experimental section

2.1. LHCII variants

Recombinant LHCII was reconstituted from a bacterially expressed protein and plant pigments. Wild type (wt) Lhcb1*2 (AB80) from *Pisum sativum* [20] and its derivatives were used, one with an additional hexahistidyl (His_6) tag at the C-terminus (wt-h) [21] and another one lacking 11 amino acids at the N-terminus (ΔN11) [22]. The derivatives ΔN11-h [23] and h-ΔN11 exhibit the same amino acid sequence as ΔN11 but contain a C-terminal and an N-terminal His_6 tag, respectively, whereas the LHCII derivative h-ΔN11-h contains both an N- and a C-terminal His_6 tag. The plasmids coding for h-ΔN11 and for h-ΔN11-h were constructed by PCR using the plasmids coding for ΔN11 and ΔN11-h, respectively, and primers introducing an N-terminal His_6 tag. The derivative Z8-ΔN11 consists of the same amino acid sequence as ΔN11 but contains, at its N terminus, a ZnS specific binding tag called Z8 [24]. The LHCII variant 4-Cys consists of the same amino acid sequence as the wild type LHCII except of a tetra-cysteine motif (Cys-Cys-Pro-Gly-Cys-Cys) between amino acids 8 and 9. Both the Z8 and the 4-Cys were produced as described for h-ΔN11 and h-ΔN11-h. A scheme of the used LHCII variants is presented in Fig. 1.

2.2. Preparation of recombinant LHCII trimers

The proteins were expressed in *Escherichia coli* as described earlier [22]. Total pigment extract, chlorophylls *a* and *b*, and carotenoids were isolated from pea thylakoids [25]. LHCII apoproteins were reconstituted with pigments to form monomeric LHCII by the detergent exchange method [19], and trimerization was carried out by affinity chromatography for His_6 tag-containing LHCII versions [26] or by trimerization in liposomes according to [23] for LHCII versions without His_6 tag.

For separating monomers and trimers and for removing unbound pigments and unfolded protein, the reconstituted complexes were ultracentrifuged through a sucrose density gradient as described earlier [27] but with a modified buffer (50 mM sodium-phosphate, pH 8.5, 0.1% (w/v) dodecyl-maltoside, 0.6 M sucrose). The trimer band was extracted from the sucrose gradient after centrifugation and its spectroscopic properties were checked by absorption, fluorescence, and CD measurements [22]. The recombinant LHCII complexes contained per

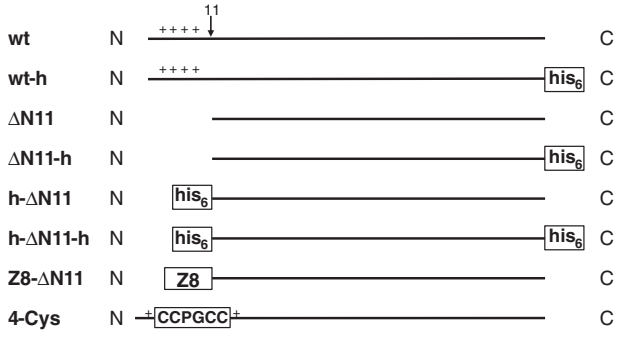

Fig. 1. Schematic sketch of the different LHCII variants used in this work. N and C denote the N and C termini of the proteins. The arrow marked 11 indicates position 11 in the polypeptide chain. The boxed-in segments represent QD-binding affinity tags as explained in the text. The series of "+" designates the N-proximal cluster of positive charges in the wt LHCII protein.

two lutein molecules about 14 chlorophylls, one neoxanthin and substoichiometric amounts of violaxanthin [28].

2.3. Synthesis of water-soluble CdTe/CdSe/ZnS quantum dots

Type-II [29–31] CdTe/CdSe/ZnS quantum dots were synthetized as described elsewhere [23]. The QDs were transferred from toluene into the aqueous phase by ligand exchange reaction with dihydrolipoic acid (DHLA). The concentration of the QDs was taken to correspond to that of the CdTe cores before shell growth, assuming there were no decomposition and no new nucleation of CdTe cores during shell growth. The phase transfer yield was close to 100%.

2.4. Assembly of LHCII and QDs

For titrating LHCII binding to QDs, both components were mixed on ice with tris-(2-carboxyethyl) phosphine (TCEP, 2 mM) and buffer (50 mM sodium-phosphate, pH 8.5, 0.1% (w/v) dodecyl-maltoside, 0.6 M sucrose) to a final volume of 50 μl (centrifugation experiments) or 500 μl (FRET experiments). The QD concentration was kept constant at 0.2 μM (centrifugation experiments) or at 15 nM (FRET experiments) whereas the concentration of the LHCII trimer varied to arrive at molar ratios between 0.5 and 5. The mixtures were incubated for 90 min on ice.

2.5. Ultracentrifugation experiments

Ultracentrifugation experiments have been carried out with a Beckmann Airfuge (rotor: A-110, Beckman Instruments, Munich). The LHCII–QD samples (see Section 2.4) were placed on top of a 130 μl sucrose cushion (1 M sucrose, 50 mM sodium-phosphate, pH 8.5, 0.1% (w/v) dodecyl-maltoside) and centrifuged at 190,000 *g* for 20 min at RT. Then 50 μl of the bottom fraction (with the LHCII–QD pellet) and subsequently 130 μl of the supernatant above the cushion (only LHCII) each were made up to a final volume of 500 μl in the same buffer containing 2 mM TCEP. The samples with the pellet fractions were sonified for 3 min in a bath sonifier (Bandelin, Germany) to suspend the LHCII–QD pellet completely. For determining the LHCII amounts in each sample absorption spectra were recorded.

2.6. Spectroscopic characterization of QDs, LHCII, and hybrid complexes

UV–vis absorption spectra were measured at RT by using an Omega-20 spectrometer (Bruins Instruments) or a V550 UV–vis spectrophotometer (Jasco, Germany). The concentration of LHCII was determined by using an extinction coefficient of $\varepsilon(670\ \text{nm}) = 1{,}638{,}000\ \text{mol}^{-1}\ \text{L}\ \text{cm}^{-1}$ [32].

Photoluminescence (PL) spectra were recorded with a Fluoromax-2 spectrometer (Jobin Yvon, Germany). Spectra were corrected for the wavelength-dependent sensitivity of the fluorometer. The QDs fluorescence quantum yields were estimated by comparing their fluorescence intensity with that of LHCII solutions possessing the same optical density at the excitation wavelength. The LHCII fluorescence quantum yield was determined to be 0.2 as described earlier [28]. For energy transfer calculations, emission spectra of LHCII–QD adducts were fitted by a sum of the individual emission spectra of LHCII and QDs, and the fitted individual spectra were integrated [23].

3. Results and discussion

3.1. Ultracentrifugation experiments are useful for comparing the binding strengths of QD-binding protein tags

For comparing the affinities of different QD-binding protein tags, several LHCII variants (Fig. 1) were generated and mixed with QDs. In this work, type-II CdSe/CdTe/ZnS core–shell QDs coated with